Virology 407 (2010) 289–295

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Tethered virions are intermediates in the assembly and release of HIV-1 particles

Alexey Karetnikov [1], Maarit Suomalainen *

Department of Virology, Haartman Institute, PO Box 21, FIN-00014 University of Helsinki, Finland

ARTICLE INFO

Article history:
Received 29 May 2010
Returned to author for revision 22 June 2010
Accepted 20 August 2010
Available online 20 September 2010

Keywords:
HIV-1
CD317
Tetherin

ABSTRACT

Vpu enhances HIV-1 release by suppressing CD317-mediated tethering of virions to the cell surface. In HeLa H1 cells, Vpu(+) infection produces significant amounts of tethered virions, although efficient virus release requires Vpu. We have analyzed membrane targeting and assembly of newly synthesized Gag in infected HeLa H1 cells by quantitative pulse-chase assays in both Vpu(+) and ΔVpu virus backgrounds and in the presence and absence of CD317. Our results show that formation of tethered virions precedes release of viruses to the culture medium in the Vpu(+) infection, and CD317 knockdown reduces tethering in both Vpu(+) and ΔVpu virus backgrounds. Significantly, our results indicate that tethered Vpu(+) viruses represent precursors for extracellular viruses, and Vpu thus appears to reverse tethering in HeLa H1 cells after budding.

Introduction

Progeny virions of human immunodeficiency virus type 1 (HIV-1) assemble at the plasma membrane (PM) of the infected cell (Deneka et al., 2007; Finzi et al., 2007; Hübner et al., 2009; Ivanchenko et al., 2009; Jouvenet et al., 2008; Jouvenet et al., 2006; Welsch et al., 2007). The structural framework of the internal virion core is formed by the viral core protein precursor Gag ($Pr55^{Gag}$). Gag proteins are synthesized on free polysomes in the cytosol and targeted to the PM posttranslationally. $Pr55^{Gag}$ binds to the cytoplasmic leaflet of the PM via an amino-terminal dual motif that consists of a myristic acid modification and a cluster of basic residues (Bryant and Ratner, 1990; Ono and Freed, 1999; Zhou et al., 1994). The HIV-1 core structure is formed at the cell surface, concomitantly with the budding process. The assembly and budding event activates viral protease present in minor core component, the Gag-Pol precursor (Kaplan et al., 1994). The protease cleaves $Pr55^{Gag}$ into matrix, capsid (CA/p24), nucleocapsid, and p6 proteins, as well as two small spacer peptides. Although viral core precursors (Gag, or Gag and Gag/Pol) assemble into extracellular enveloped virus-like particles (VLPs) in the absence of other virus components (Gheysen et al., 1989), virus release from certain restrictive cell types, such as HeLa, Jurkat, and peripheral blood mononuclear cells, is more efficient if core components are expressed together with the viral accessory protein Vpu (Göttlinger et al., 1993; Klimkait et al., 1990; Neil et al., 2007). Vpu enhances virus release from these restrictive cell types by counteracting a host cell factor CD317/BST2/HM1.24/tetherin (referred to as CD317 in this work) (Neil et al., 2008; Neil et al., 2007; van Damme et al., 2008). In the absence of Vpu, progeny virions form at the PM of restrictive cells, but the newly formed virions remain attached to the cell surface by protein tethers formed by homodimers or higher-order oligomers of CD317 (Hammonds et al., 2010; Perez-Caballero et al., 2009). The tethered ΔVpu viruses can be released from restrictive cells by protease treatment of intact cells (Neil et al., 2007). Antivirus activity of CD317 is not limited to HIV-1. The protein inhibits release of many different retroviruses and also viruses belonging to the families *Arenaviridae* and *Filoviridae* (Jouvenet et al., 2009; Kaletsky et al., 2009; Sakuma et al., 2009), as well as Kaposi's sarcoma-associated herpesvirus (Mansouri et al., 2009). CD317 is a protein with an unusual topology, since it has both an N-terminal transmembrane domain and a C-terminal glycosyl-phosphatidylinositol (GPI) anchor, and an extracellular domain capable of forming a disulfide-linked coiled coil structure (Hinz et al., 2010). Both of the membrane anchors as well as the extracellular disulfide-linked coiled coil are needed for antiviral activity against HIV-1 (Andrew et al., 2009; Goffinet et al., 2009; Hinz et al., 2010; Iwabu et al., 2009; Perez-Caballero et al., 2009). Apparently, only the overall configuration of CD317 is important for its antiviral activity, since an artificially constructed CD317-like protein with no sequence homology to CD317 restricts release of HIV-1 if the protein is incorporated into the envelope of the budding virus particle (Perez-Caballero et al., 2009). The exact mechanism by which CD317 tethers newly formed HIV-1 virions to the cell surface, or the molecular mechanism by which Vpu counteracts CD317, is still unclear. CD317 can infiltrate the forming HIV-1 envelope (Fitzpatrick et al., 2010; Habermann et al., 2010; Hammonds et al., 2010; Perez-Caballero et al., 2009), and Vpu-mediated exclusion of CD317 from virus assembly/budding sites could be sufficient to neutralize antiviral activity of CD317. Indeed, several reports have

* Corresponding author. Present address: University of Zurich, Institute of Molecular Life Sciences, Winterthurerstr. 190, 8057 Zurich, Switzerland. Fax: +41 44 6356822.
E-mail address: maarit.suomalainen@imls.uzh.ch (M. Suomalainen).
[1] Present address: Department of Biochemistry and Goodman Cancer Research Centre, McGill University, 1160 Avenue Des Pins Ouest, Cancer Pavilion, Room #609, Montréal, Québec, Canada H3A 1A3.

0042-6822/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2010.08.020

shown that Vpu can downmodulate CD317 from the cell surface in different cell lines, as well as cause decline in steady-state levels of CD317 (Andrew et al., 2009; Douglas et al., 2009; Goffinet et al., 2009; Gupta et al., 2009; Habermann et al., 2010; Mangeat et al., 2009; McNatt et al., 2009; Mitchell et al., 2009; Rong et al., 2009; Sato et al., 2009; Schindler et al., 2010; van Damme et al., 2008). However, two recent reports indicate that CD317 is found in both Vpu(+) and ΔVpu virions (Fitzpatrick et al., 2010; Habermann et al., 2010). Furthermore, Vpu enhances virus release from infected CEMx174 and H9 cells without causing down-regulation of cell surface CD317 (Miyagi et al., 2009).

In the present study, we have used quantitative pulse-chase assays to analyze the effects of Vpu and CD317 on membrane targeting and incorporation of newly synthesized $Pr55^{Gag}$ into virions in infected HeLa H1 cells. These cells produce considerable amounts of surface-tethered HIV-1 particles even when Vpu is expressed in the infected cell (Harila et al., 2006), and the amount of tethered viruses could be reduced by knockdown of CD317 in both Vpu(+) and ΔVpu virus backgrounds. Surprisingly, our data suggest that tethering is an intermediate step in the release of Vpu(+) virions.

Results

Membrane targeting and assembly of newly synthesized Gag in HeLa H1 cells infected with Vpu(+) or Vpu-deleted HIV-1

Vpu is required for efficient release of progeny HIV-1 virions from infected HeLa H1 cells, but significant amounts of produced virions remain tethered to the cell surface even if Vpu is expressed in the infected cells (e.g. EM analysis in Harila et al., 2006). Fig. 1 shows a comparison of Vpu(+) and ΔVpu virus infections in HeLa H1 cells [NL4-3(ΔEnv) and NL4-3(ΔVpu/ΔEnv), respectively; both viruses are lacking the *env* gene]. Cells were infected with VSV-G-pseudotyped Vpu(+) or ΔVpu virus at the same MOI (~5 infectious units/cell), and at 24 h after infection, cells were metabolically labeled with [^{35}S] methionine for 30 min and chased for up to 3 h. These time points were chosen because pilot experiments indicated that virus production had reached maximal level at 24 h after infection, and kinetic analysis indicated that ~80% of maximal incorporation of labeled Gag into VLPs was achieved during the first 3 h of chase (data not shown). After chase, the sample was divided into three fractions: (1) VLPs

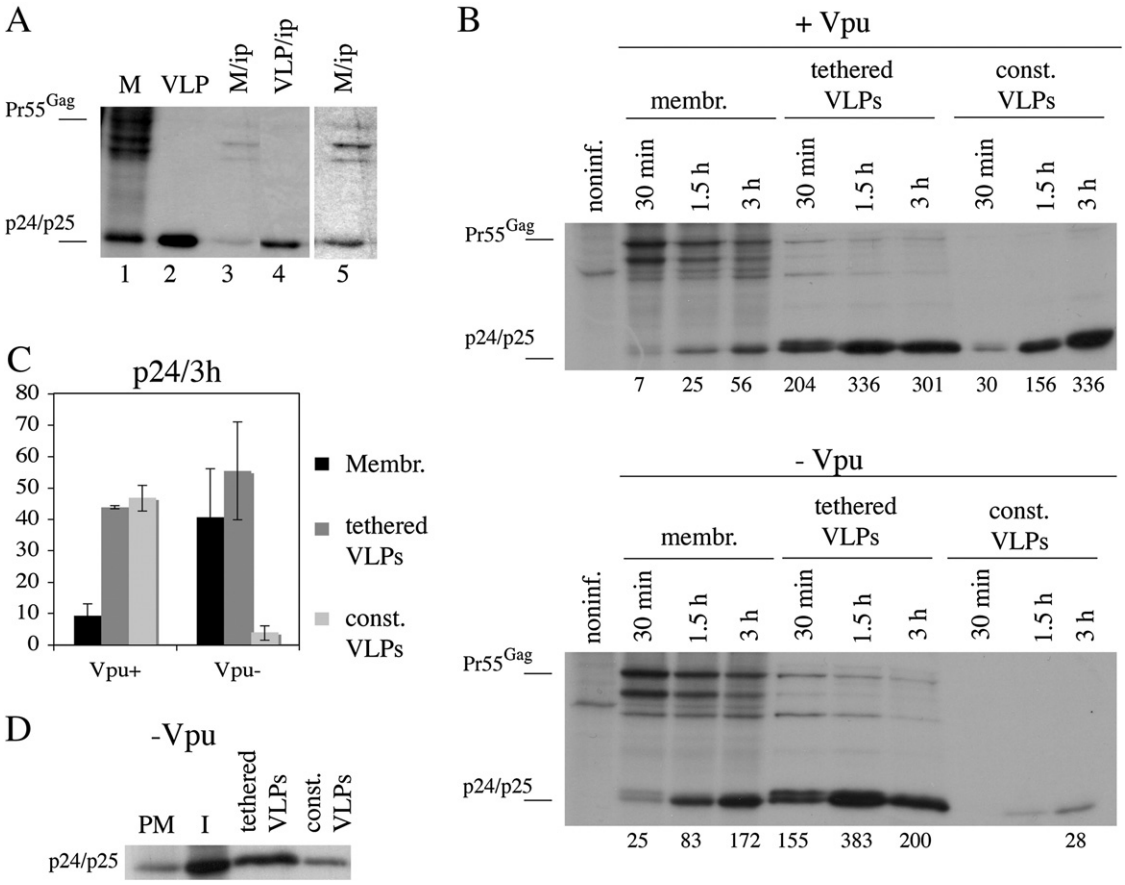

Fig. 1. Comparison of Vpu(+) and ΔVpu virus infections. (A) Labeled Gag-derived bands are major bands in the total membrane and particulate medium fractions of infected HeLa H1 cells. NL4-3(ΔEnv)-infected cells were metabolically labeled with [^{35}S]methionine for 30 min and chased for 3 h. Shown are total membrane fraction of cell extracts (lane 1), particulate fraction of culture medium (=constitutively released VLPs; lane 2), anti-CA immunoprecipitation of the total membrane fraction (lanes 3 and 5), and anti-CA immunoprecipitation of the particulate fraction of culture medium (lane 4). Lanes 3 and 5 are otherwise identical except that lane 5 has been enhanced and contrasted differently in Photoshop to better visualize the $Pr55^{Gag}$ and its early processing intermediates which were not as efficiently immunoprecipitated by the antibody as p24. Positions of $Pr55^{Gag}$ and p24/25 are shown on the left. (B) Infected HeLa H1 cells were pulsed for 30 min and chased for the indicated times. Shown are virus particles recovered from culture supernatants (=const. VLPs, or constitutively released VLPs), virus particles released from cells after trypsin-treatment (=tethered VLPs), and the amount of Gag remaining in membranes after trypsin treatment (=membr.). Equal aliquots of the different fractions were analyzed by SDS–PAGE without prior immunoprecipitation. +Vpu indicates NL4-3(ΔEnv) infection, and −Vpu indicates NL4-3(ΔVpu/ΔEnv) infection. The first lanes (noninf.) show a membrane fraction isolated from noninfected cells. Positions of $Pr55^{Gag}$ and p24/25 are shown on the left. Quantitation of p24/p25 bands (arbitrary values) are shown at the bottom of panels. (C) Percentage of labeled p24/p25 in the membrane-, tethered VLPs- and constitutively released VLPs-fractions at the 3-h chase point. The values represent mean values from three independent experiments with standard deviations shown. (D) p24 in the membrane fraction of NL4-3(ΔVpu/ΔEnv) homogenates represents protein present in internal membranes. NL4-3(ΔVpu/ΔEnv)-infected cells were pulsed for 30 min and chased for 3 h. Trypsin-treated cells were coated with silica beads before homogenization, and cell homogenates were fractionated into PM and internal membranes (I). Only the p24/p25 part of the gel is shown.

constitutively released into the culture supernatant, (2) VLPs tethered to the cell surface (=VLPs released after trypsin treatment of cells), and (3) membrane-associated material. Fractions 1 and 2 were purified by ultracentrifugation through a 20% sucrose cushion, whereas fraction 3 was isolated from postnuclear supernatants of cell homogenates prepared after trypsin treatment of cells. The postnuclear supernatants were fractionated on a Nycodenz step gradient to separate membranes from cytosol. Equal aliquots of the three fractions were analyzed by gel electrophoresis. As shown in Fig. 1A, labeled Gag proteins are major proteins in the membrane fraction of infected HeLa H1 cells when MOI ~5 infectious units/cell is used, and thus the VLP and membrane fractions can be analyzed without prior immunoprecipitation. The upper panel in Fig. 1B shows the analysis of Vpu(+) infection. The precursor $Pr55^{Gag}$, and a partially processed intermediate of $Pr55^{Gag}$, could be identified in the membrane fraction after 30 min of chase. The amounts of these Gag forms were reduced at later time points, and labeled p24 became evident in the membrane fraction. Interestingly, labeled p24 accumulated in tethered VLPs more rapidly than in constitutively released VLPs; the amount of labeled p24 in tethered VLPs leveled off after 1.5 h of chase, whereas there still was a clear increase in the amount of labeled p24 in constitutively released VLPs between 1.5- and 3-h chase. Quantitations from three independent experiments indicated that the Vpu(+) infection had roughly equal amounts of tethered vs. constitutively released VLPs at the 3-h chase point (Fig. 1C). The lower panel in Fig. 1B shows analysis of NL4-3(ΔVpu/ΔEnv)-infected cells. Efficient constitutive release of virus particles from HeLa H1 cells is dependent on Vpu, and only trace amounts of labeled p24 were recovered from the culture supernatants of NL4-3 (ΔVpu/ΔEnv)-infected cells: quantitation from three independent experiments showed that constitutively released VLPs harbored only ~4% of total labeled p24 at the 3-h chase point (Fig. 1C). Tethered VLPs with labeled p24 formed with similar kinetics as in the NL4-3 (ΔEnv) infection. The amounts of labeled Gag precursors in the membrane fractions after 30-min chase were similar in the NL4-3 (ΔEnv) and NL4-3(ΔVpu/ΔEnv) infections, but at later time points, more labeled p24 was seen in the membrane fractions of NL4-3 (ΔVpu/ΔEnv)-infected cells than in cells infected with the Vpu(+) virus. This most likely reflected endocytosis of tethered VLPs in the ΔVpu background (Harila et al., 2006; Harila et al., 2007), since if the plasma membrane of trypsin-treated cells (3 h chase point) was coated with silica beads prior to homogenization, and the homogenate was fractionated on Nycodenz gradient into silica-coated plasma membrane and uncoated internal membrane fractions (Harila et al., 2006), the majority of trypsin-resistant cell-associated labeled p24 fractionated with internal membranes (Fig. 1D). Endocytosis of tethered VLPs would also explain the apparent decrease of labeled p24 in tethered VLPs between the 1.5-h and 3-h chase points in the ΔVpu infection (Fig. 1B). However, the efficiency of endocytosis of tethered VLPs was somewhat different in repetitive experiments, as shown by the variations in the relative amounts of labeled p24 in membrane vs. tethered VLPs fractions in ΔVpu infection at the 3-h chase point (Fig. 1C). The total amount of labeled p24 (i.e., sum of p24 in the membrane fraction, tethered VLPs, and constitutively released VLPs) can be used as a rough estimate for the overall assembly efficiency, since processing of $Pr55^{Gag}$ is closely linked to budding (Kaplan et al., 1994). In the experiment shown in Fig. 1B, the total amount of labeled p24 at the 3-h chase point in NL4-3(ΔVpu/ΔEnv)-infected cells was 58% of that of the Vpu(+) infection. However, if crude cell homogenates were used for membrane isolation instead of postnuclear supernatants, total p24 in NL4-3(ΔVpu/ΔEnv)-infected cells after 3 h of chase was over 80% of the equivalent p24 of NL4-3(ΔEnv) infection (data not shown). This difference most likely reflects loss of the PM and/or internal membranes into the pellet fraction in the centrifugation step used to prepare the postnuclear supernatants.

Knockdown of CD317 reduces tethering of both Vpu(+) and ΔVpu virions

We next tested the effect of CD317-specific siRNAs to determine whether tethering of progeny VLPs to the cell surface in Vpu(+) and ΔVpu virus backgrounds was due to CD317. Immunofluorescence staining of siRNA- and mock-transfected cells (=cells treated only with the transfection reagent) with anti-CD317 antibody indicated that the CD317 siRNAs effectively reduced intracellular amounts of CD317 (Fig. 2A). In the experiment shown in Fig. 2B, cells were transfected with CD317 siRNAs or mock-transfected, infected with VSV-G-pseudotyped NL4-3(ΔVpu/ΔEnv) the following day, and analyzed at 24 h after infection. The cells were metabolically labeled for 30 min, and chased for 30 min, 2 h, or 3 h. Crude cell homogenates were used for the isolation of membrane fraction. The CD317 siRNAs dramatically increased constitutive release of VLPs from NL4-3(ΔVpu/ΔEnv)-infected cells and reduced the amounts of labeled p24 in membrane fraction and tethered VLPs.

The effect of CD317 knockdown on virus production in NL4-3 (ΔEnv)-infected HeLa H1 cells is shown in Fig. 2C. The cells were metabolically labeled for 30 min at 24 h after infection and chased for 3 h. The knockdown of CD317 decreased amounts of tethered VLPs in the Vpu(+) virus background. This reduction in tethered VLPs was specific to CD317 siRNAs since similar amounts of a control nontargeting siRNA, siRNAs directed against CAML (calcium signal-modulating cyclophilin ligand) or several other targeting siRNAs (e.g., siRNAs against CD44, CD63, CD9; data not shown) did not reduce the amounts of tethered VLPs in Vpu(+) infection. Quantitations from four different Vpu(+) infection experiments indicated a statistically significant decrease in relative amounts of tethered VLPs in CD317 siRNA-treated cells in comparison to mock- or control siRNA-treated cells (Fig. 2D). The difference between mock- and control siRNA-treated cells was not statistically significant.

Tethered VLPs are precursors for constitutively released VLPs

Since labeled p24 appeared in tethered and constitutively released VLPs with different kinetics (Fig. 1), this suggested that these two VLP populations were either formed by different pathways or that tethered VLPs represented precursors for constitutively released VLPs. In Fig. 3, we have tested this latter possibility. Our objective was to determine whether enzymatic removal of tethered VLPs would compromise subsequent release of labeled p24 into culture medium. NL4-3(ΔEnv)-infected cells were metabolically labeled for 30 min, and treated with trypsin at the 1.5- or 2-h chase point to remove tethered VLPs. Control cells on parallel plates received only a media change. Cells were then incubated for further 2 h before constitutively released and tethered VLPs were collected. To ascertain that virus production was compared from similar numbers of cells, the levels of glycerol aldehyde-3-phosphate dehydrogenase (GAPDH) in cell extracts of trypsin-treated and control cells were analyzed on Western blots, and used as a normalization factor for cell numbers (data not shown). Fig. 3A shows the analysis of cells treated with trypsin at the 2-h chase point. At this chase point, the cells had considerable amounts of tethered VLPs. Control cells that received only a change of culture medium at the 2-h chase point efficiently released VLPs during next 2 h of chase. In contrast, very little labeled p24 was found in constitutively released or tethered VLPs at the 4-h chase point in the sample that had been treated with trypsin at the 2-h chase point. In two separate experiments, the trypsin treatment at the 1.5- or 2-h chase point (=experiment shown in Fig. 3A) reduced subsequent constitutive release of VLPs by 77% or by 75%, respectively, in comparison to control, nontreated cells. The sum of tethered and constitutively released viruses from the trypsin-treated cells was ~16%–18% of that of control cells in the two experiments. However, if the amount of labeled p24 removed by trypsin at the 1.5- or 2-h chase point

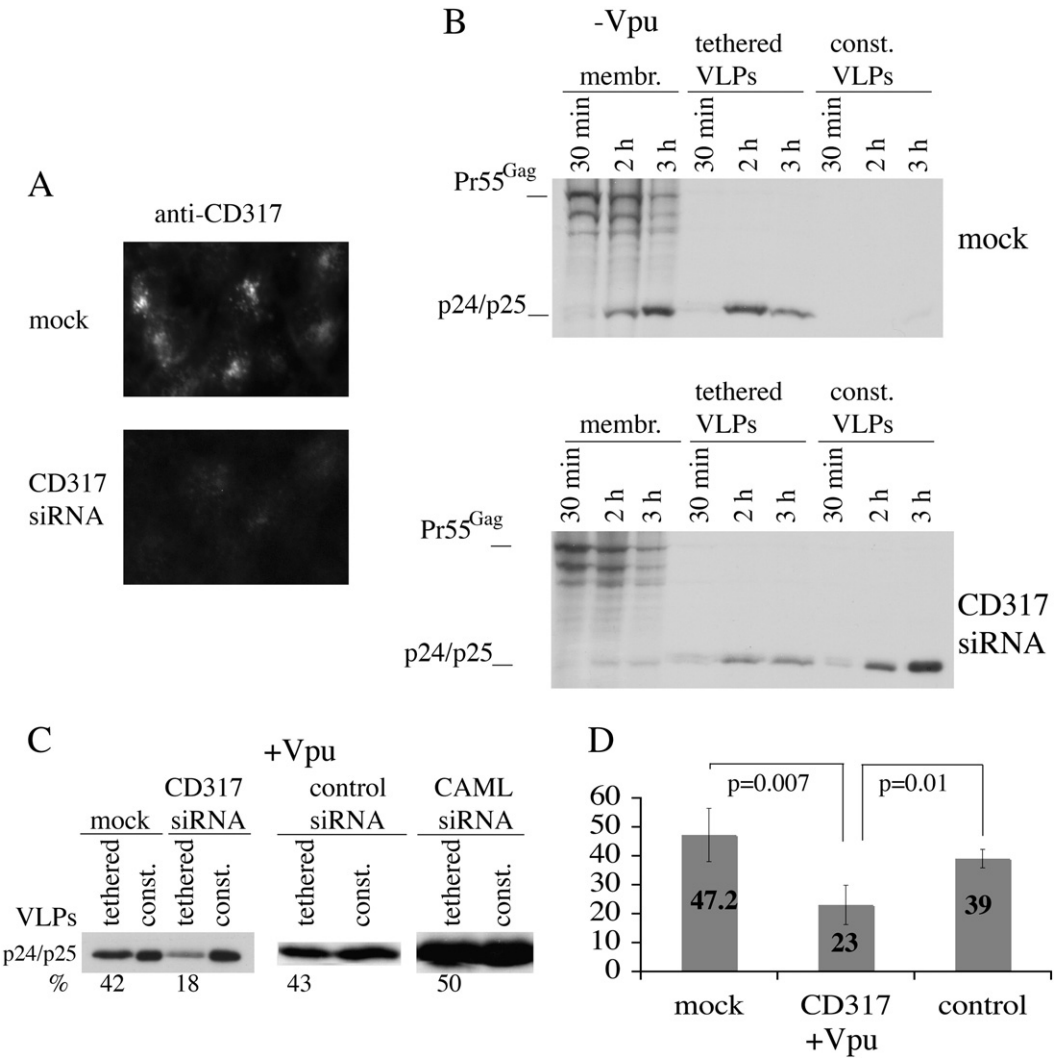

Fig. 2. CD317 knockdown. (A) CD317 siRNAs efficiently reduce intracellular levels of CD317. Cells were either mock-transfected or transfected with CD317-specific siRNAs prior to infection with NL4-3(ΔEnv). At 48 h after transfection and 24 h after infection cells were fixed, permeabilized and stained with mouse polyclonal anti-CD317 antibodies and an Alexa555-conjugated anti-mouse antibody. (B) HeLa H1 cells infected with ΔVpu virus. Cells were either mock-transfected or transfected with CD317-specific siRNAs prior to infection with NL4-3(ΔVpu/ΔEnv). The cells were metabolically labeled for 30 min, chased for the indicated times, and analyzed as described in the legend for Fig. 1. (C) VLP production from mock-, CD317 siRNA-, control nontargeting siRNA- and CAML siRNA-treated HeLa H1 cells infected with Vpu(+) virus. Cells were metabolically labeled for 30 min and chased for 180 min. Mock and CD317 siRNA results are from the same experiment, whereas control and CAML siRNA results are from separate experiments. Percentage of labeled p24/p25 in tethered VLPs is shown at the bottom of the panel. Only the p24/p25 part of the gel is shown. (D) Statistical analysis of virus production from mock-, control siRNA or CD317 siRNA-transfected cells in Vpu(+) infection. Shown is the percentage of labeled p24/p25 in tethered VLPs at the 3-h chase point. The values represent mean values from four independent experiments with standard deviations. *p* values are from Student's *t* test.

is taken into account, then the total amount of labeled p24 of the trypsin-treated sample at the 4-h chase point was 87%–89% of that of the respective control samples. To verify that trypsin treatment did not cause general inhibition of virus release, we performed trypsin treatment after the 30-min pulse and chased cells for 2 h. As shown in Fig. 3B, in this case, the trypsin-treated cells produced virus as efficiently as control nontreated cells. In two separate experiments, the constitutive virus release from cells treated with trypsin immediately after the pulse was 112% or 98% of that of control cells, and the total amount of labeled p24 in tethered VLPs and constitutively released VLPs of the trypsin-treated cells was 83% or 71% of that of control cells. Thus, trypsin treatment of cells did not cause a general inhibition of virus release. The trypsin-treated cells had 60%–78% less tethered VLPs than the control cells when trypsin treatment was done immediately after the pulse. This was most likely due to the fact that trypsin treatment removed tethering factors from the plasma membrane and 2 h of chase was too short a time to effectively repopulate the cell surface with these factors. Suppression of tethering after trypsin treatment was evident also in ΔVpu-background, since if NL4-3(ΔVpu/ΔEnv)-infected cells were trypsin-treated after a 30-min pulse, and chased for 2 h, the cells efficiently released VLPs into the culture medium (Fig. 3C). This effect was observed in two separate experiments. In Fig. 3C, the sum of labeled p24 in tethered and constitutively released VLPs of the trypsin-treated cells was 1.7-fold higher than that of control cells. The lower value of control cells was most likely due to endocytosis of tethered VLPs. Taken together, the results in Fig. 3 imply that tethered VLPs are precursors for constitutively released VLPs, and that 'tethering' is an intermediate step in the formation and release of Vpu(+) viruses.

Discussion

CD317/tetherin is an antiviral defense factor that has a capacity to tether newly formed HIV-1 virions to the cell surface (Neil et al., 2008; van Damme et al., 2008). These tethered virions can be released from cell surface by treatment with a protease. Vpu counteracts this effect of CD317/tetherin, and Vpu is needed for efficient 'constitutive' virus release from cells that express CD317, such as the HeLa H1 cells used in the present study. Previous EM studies suggested that these cells

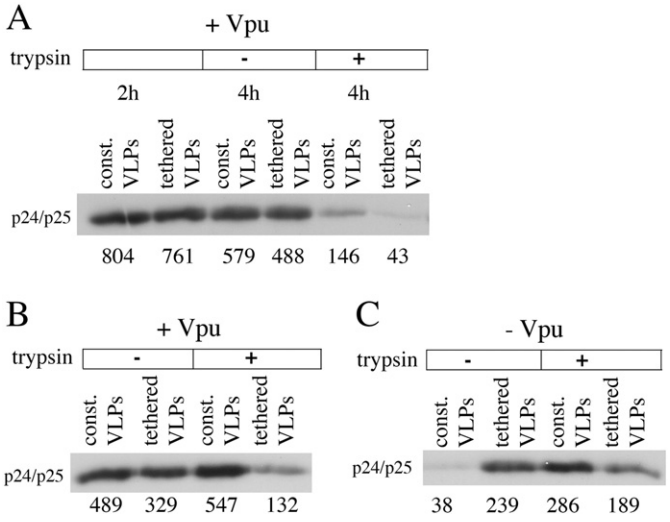

Fig. 3. Tethered (Vpu+)–VLPs are precursors for extracellular VLPs. (A) Removal of tethered VLPs at the 2 h chase point significantly reduces constitutive virus release during the next 2 h of chase. NL4-3(ΔEnv)-infected HeLa H1 cells on three parallel plates were metabolically labeled with [^{35}S]methionine for 30 min, and chased for 2 h. At the 2 h chase point, one plate was used to determine the amounts of constitutively released and tethered VLPs at this time point (=2 h), whereas cells on the two remaining plates either received a medium change (4 h/− trypsin) or were treated with trypsin (4 h/+ trypsin) to remove tethered VLPs, and then chased for further 2 h. At the end of this second 2-h chase period, the amounts of constitutively released and tethered VLPs were again analyzed. Only the p24/p25 part of the gel is shown, and quantifications of p24/p25 bands (arbitrary values) are shown at the bottom of the panel. (B) Trypsin treatment does not cause general inhibition of virus release. NL4-3 (ΔEnv)-infected HeLa H1 cells were metabolically labeled with [^{35}S]methionine for 30 min. After the pulse, cells were either treated with trypsin or not treated, and chased for 2 h, and the amounts of constitutively released and tethered VLPs were analyzed. (C) Trypsin treatment enhances constitutive virus release from NL4-3(ΔVpu/ΔEnv)-infected HeLa H1 cells. Cells were metabolically labeled and analyzed as in B.

produce considerable amounts of tethered virions even when Vpu is expressed in the virus-producing cells (Harila et al., 2006), and this was confirmed by quantitative pulse-chase assays and subcellular fractionations of the present study. We analyzed targeting of newly synthesized Gag proteins to membranes in infected HeLa H1 cells and compared the membrane targeting to formation of tethered and constitutively released VLPs. The analyses were done in both Vpu(+) and ΔVpu virus backgrounds [NL4-3(ΔEnv) and NL4-3(ΔVpu/ΔEnv) infections, respectively]. Our results indicated that the tethered and constitutively released VLPs of Vpu(+) infection formed with different kinetics, whereas the amount of labeled tethered VLPs reached maximal levels after about 1.5 h of chase, the amount of labeled virions in the culture medium still increased after this chase point. Tethered VLPs in the ΔVpu background formed with similar kinetics as in the Vpu(+) infection, but virus release into the medium was inefficient in the absence of Vpu. Interestingly, when tethered VLPs at the 2-h chase point were removed from NL4-3(ΔEnv)-infected cells by trypsin, this dramatically reduced the amount of virus released into the culture medium during next 2 h of chase. In contrast, control non-trypsin-treated cells efficiently accumulated virus into the culture medium between 2 h and 4 h of chase. Furthermore, if trypsin treatment was done immediately after the pulse, both NL4-3 (ΔEnv)- and NL4-3(ΔVpu/ΔEnv)-infected cells efficiently produced labeled VLPs into the culture medium during the following 2 h of chase, thus indicating that trypsin treatment in general was not inhibitory for virus release. These results imply that tethered virions in the Vpu(+) background represented precursors for extracellular viruses and that tethering was an intermediate step in the release of Vpu(+) virions to the culture medium. A reversible tethering mechanism predicts a lag time for particle release after completed assembly and budding. Indeed, such a lag time has been observed in live analyses of HIV-1 particle formation (Ivanchenko et al., 2009), although at present it is not known whether the lag time is due to a budding-related phenomenon (e.g., membrane fission–reaction) or to some postbudding event.

But what is the tethering mechanism in the Vpu(+) infection in HeLa H1 cells? As discussed above, virus tethering in the ΔVpu-background is mediated by CD317, and CD317 promotes virus tethering by directly cross-linking newly formed virions to the cell surface (Fitzpatrick et al., 2010; Hammonds et al., 2010; Perez-Caballero et al., 2009). Tethering of ΔVpu virions to the cell surface of infected HeLa H1 cells is similar to tethering of ΔVpu virions described in other cells, since knockdown of CD317 in infected HeLa H1 cells greatly augmented release of ΔVpu–VLPs into the culture medium. The CD317 siRNAs also reduced the amount of tethered VLPs in the Vpu(+) infection, and this reduction was specific for CD317 siRNAs since several other targeting or nontargeting siRNAs that we have tested have not given the same effect as the CD317-specific siRNA pool used in the present study. This suggests that tethering of VLPs in the Vpu(+) background is, directly or indirectly, CD317-mediated as well. This is also supported by the observation that tethered VLPs formed with similar kinetics in both Vpu(+) and ΔVpu virus backgrounds. In the experimental setup used, we could not do the control experiment to express siRNA-resistant CD317 in the knock-down cells, because we have not yet found a transfection reagent that would efficiently transfect plasmid DNA into HeLa H1 cells. Exactly how CD317 tethers nascent HIV-1 particles to the cell surface, or how Vpu counteracts CD317, is still unknown. Both the transmembrane domain and the carboxy-terminal GPI anchor, as well as ability to form homodimers are important for antiviral activity of CD317 (Andrew et al., 2009; Goffinet et al., 2009; Hinz et al., 2010; Iwabu et al., 2009; Perez-Caballero et al., 2009), but the configuration of CD317 in virus tethers is unclear. CD317 can be incorporated into the forming HIV-1 envelope (Fitzpatrick et al., 2010; Habermann et al., 2010; Hammonds et al., 2010; Perez-Caballero et al., 2009), and two models have been proposed for CD317-mediated tethering mechanism. One model postulates that ectodomain interactions between virion- and plasma membrane-associated CD317 proteins form protein tethers that keep newly formed virions attached to the PM, whereas the other model predicts that the two membrane anchors of CD317 partition into separate membranes during budding, i.e., one end of CD317 becomes embedded in the donor plasma membrane and the other end in the virion envelope (e.g., Perez-Caballero et al., 2009). Although recent structural analysis of CD317 indicated that the protein is capable of adopting an extended rod-like conformation compatible with the latter tethering model (Hinz et al., 2010), at present there is no direct evidence for either model. For example, Fitzpatrick et al. (2010) reported that enzymatic cleavage of GPI anchors by phosphatidyl inositol-specific phospholipase C failed to release tethered HIV-1 virions. Furthermore, EM studies have identified long, linear filamentous strands between tethered viruses, and these structures were decorated with anti-CD317 antibodies (Hammonds et al., 2010). This implies that multiple CD317 proteins might form the tethering structures. Apparently, CD317 does not need any specific cofactors to inhibit HIV-1 release, since an artificially constructed CD317-like protein with no sequence homology to CD317, but with similar overall structure has been shown to be capable of limiting HIV-1 release (Perez-Caballero et al., 2009). In contrast, the CD317-mediated tethering mechanism of human T-cell leukemia virus type 1 (HTLV-1) is more complex since CD317 is part of a multiprotein biofilm-like structure that tethers HTLV-1 viruses to the cell surface (Pais-Correia et al., 2010). Vpu downregulates cell surface expression of CD317 in many cell types (Douglas et al., 2009; Goffinet et al., 2009; Gupta et al., 2009; Mangeat et al., 2009; Rong et al., 2009; Sato et al., 2009; van Damme et al., 2008), and this has led to the hypothesis that Vpu inhibits infiltration of virus particles by CD317 via lowering concentration of CD317 at the plasma membrane.

However, two recent studies indicated that Vpu(+) virions are not devoid of CD317. In fact, one of these studies found an apparent enrichment for CD317 in both Vpu(+) and ΔVpu virion envelopes in comparison to the PM, and only minor differences in the amounts of CD317 between the two virus populations (Habermann et al., 2010), whereas the other study found that lack of Vpu leads to about 2-fold higher concentration of CD317 in the virion envelope (Fitzpatrick et al., 2010). Thus, simple exclusion of CD317 from virus budding sites does not seem to fully account for Vpu-mediated suppression of antiviral activity of CD317. Of note, our flow cytometry analyses have indicated that Vpu reduced CD317 at the cell surface by 46% in infected HeLa H1 cells, whereas cells infected with ΔVpu virus had surface amounts of CD317 comparable to those of noninfected cells (data not shown). Exactly how important the down-modulation of CD317 from the cell surface is for enhancement of virus release by Vpu remains to be determined since in certain cell types Vpu appears to promote virus release without downregulating the surface CD317 (Miyagi et al., 2009). Our results suggest a new viewpoint into the Vpu-mediated suppression of CD317, namely that Vpu might, directly or indirectly, resolve tethered particles after budding. However, further studies are needed to substantiate this hypothesis, and to formulate a model how this putative Vpu-mediated postbudding release of tethered VLPs could function.

Materials and methods

Cell culture, viruses, and plasmid constructs

HeLa H1 and 293T cells were cultured as previously described (Harila et al., 2006; Holm et al., 2003). Production of vesicular stomatitis virus (VSV)–G-protein-pseudotyped infectious recombinant HIV-1 in 293T cells has been previously described (Harila et al., 2006). Titers of virus stocks were determined by immunofluorescence staining of infected HeLa H1 cells (anti-CA staining at 24 h after infection). Plasmid pNL4-3(ΔVpu/ΔEnv) has been previously described (Harila et al., 2007). Plasmid pNL4-3(ΔEnv) was constructed by replacing the 2680-bp SalI–BamHI fragment of pNL4-3 (Adachi et al., 1986) with the equivalent 2100-bp SalI–BamHI fragment of pNL4-3(Gag/ΔEnv) (Harila et al., 2006).

Subcellular fractionations

HeLa H1 cells on 10-cm plates were infected with VSV-G-pseudotyped recombinant HIV-1 (about 5 infectious units/cell). At ~24 h after infection, cells were metabolically labeled with [^{35}S] methionine for 30 min and chased for various times as previously described (Holm et al., 2003). After the chase, cell culture supernatants were collected, and after removal of cell debris by 200×*g* centrifugation, the cleared supernatants were layered onto a 20% sucrose cushion (wt./vol., in 25 mM HEPES pH 7.4, 150 mM NaCl, 2 mM $MgCl_2$), and centrifuged at 100,000×*g* for 60 min to obtain pellets, which represented constitutively released VLPs. To collect the tethered VLPs, cells were briefly washed with phosphate-buffered saline containing 0.02% EDTA after harvesting culture supernatants and treated with trypsin (0.5 mg/ml) at 37 °C for 8 min. The reaction was stopped by adding culture medium containing 7% fetal bovine serum, cells were pelleted and VLPs from the culture supernatants were concentrated through sucrose as described above. The cell pellets from the trypsin treatment were washed once with a buffer containing 25 mM HEPES pH 7.4, 150 mM NaCl, 2 mM $MgCl_2$, and homogenized with a tight-fitting Dounce homogenizer in the wash buffer supplemented with additional 2 mM $MgCl_2$, 20 μg of phenylmethylsulfonyl fluoride, 1 μg of CLAP inhibitor mix (chymostatin, leupeptin, aprotinin, and pepstatin), and 100 μg of soybean trypsin inhibitor per ml. Either postnuclear supernatants or crude cell homogenates (the latter treated with 1.2 mg/ml DNase I for 30 min on ice) were used for isolation of membranes. The postnuclear supernatants or DNase I-treated crude cell homogenates were mixed with 100% Nycodenz solution (Axis-Shield PoC) to give a final 60% Nycodenz concentration. The sample was overlaid with 50% Nycodenz and a buffer solution containing 25 mM HEPES pH 7.4, 150 mM NaCl, and 1 mM EDTA. The gradient was centrifuged at 100,000×*g* for 60 min, and the total membrane fraction at the 50% Nycodenz-buffer interphase (=membrane-associated material) was collected. Equal aliquots of the membrane-associated material, tethered VLPs, and constitutively released VLPs were analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE). The labeled Gag bands in membrane fractions and VLPs were quantified by Fuji BAS-1500 phosphoimager by subtracting a background value (=equal area from above or below the Gag band) from the value measured for Gag bands. The identity of Gag-derived bands in membrane and VLP fractions was verified by immunoprecipitation using a polyclonal rabbit anti-CA antibody (provided by Jorma Hinkula, Linkoping University, Linkoping, Sweden). Aliquots of postnuclear supernatants of cell extracts were analyzed by Western blotting using a polyclonal anti-glyceraldehyde-3-phosphate dehydrogenase antibody (FL-335; Santa Cruz Biotechnology, Inc.) to ensure that samples to be compared contained similar numbers of cells.

Silica coating of PM and separation of PM and internal membranes were carried out as previously described (Harila et al., 2006).

RNAi

Small interfering RNAs (siRNAs) were transfected into HeLa H1 cells (~40%–50% confluent cell cultures on 10-cm plates) by using siLentFect (BioRad; 20 μl per 10-cm plate) in accordance with the manufacturer's instructions. At about 22 h after transfection, cells were infected and analyzed at 24 h after infection. For CD317 or CAML knockdowns, HeLa H1 cells were transfected with siGENOME ON-TARGET plus SMARTpool human CD317- or CAML-specific siRNAs (Dharmacon; 120 pmol per 10-cm plate). Control cells were treated with the transfection reagent alone or with a control nontargeting siRNA (Harila et al., 2006). Efficiency of CD317 knockdown was checked by staining paraformaldehyde-fixed and saponin-permeabilized mock- or siRNA-treated cells with a mouse polyclonal anti-CD317 antibody (B02P: Abnova) and an Alexa555-conjugated goat anti-mouse antibody at 48 h after transfection. The images were acquired with Zeiss Axioplan 2 microscope and Wasabi software and processed in Photoshop.

Statistical analyses

Error bars in all the graphs represent the standard deviation of the mean with *p* values from Student's *t*-tests.

Acknowledgments

We thank Jorma Hinkula for the anti-HIV-1 CA monoclonal antibody.

This study was supported by a grant from the Academy of Finland (124235) to M.S.

References

Adachi, A., Gendelman, H.E., Koenig, S., Folks, S., Willey, R., Rabson, A., Martin, M.A., 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular retrovirus clone. J. Virol. 59, 284–291.

Andrew, A.J., Miyagi, E., Kao, S., Strebel, K., 2009. The formation of cysteine-linked dimers of BST-2/tetherin is important for inhibition of HIV-1 virus release but not for sensitivity to Vpu. Retrovirology 6, 80.

Bryant, M., Ratner, L., 1990. Myristoylation-dependent replication and assembly of human immunodeficiency virus 1. Proc. Natl. Acad. Sci. U. S. A. 87, 523–527.

Deneka, M., Pelchen-Matthews, A., Byland, R., Ruiz-Mateos, E., Marsh, M., 2007. In macrophages, HIV-1 assembles into an intracellular plasma membrane domain containing the tetraspanins CD81, CD9 and CD53. J. Cell Biol. 177, 329–341.

Douglas, J.L., Viswanathan, K., McCarroll, M.N., Gustin, J.K., Früh, K., Moses, A.V., 2009. Vpu directs the degradation of the human immunodeficiency virus restriction factor BST-2/tetherin via a betaTrCP-dependent mechanism. J. Virol. 83, 7931–7947.

Finzi, A., Orthwein, J., Mercier, J., Cohen, E.A., 2007. Productive human immunodeficiency virus type 1 assembly takes place at the plasma membrane. J. Virol. 81, 7476–7490.

Fitzpatrick, K., Skasko, M., Deerinck, T.J., Crum, J., Ellisman, M.H., Guatelli, J., 2010. Direct restriction of virus release and incorporation of the interferon-induced protein BST-2 into HIV-1 particles. PLoS Pathog. 6 (3), e1000701.

Gheysen, D., Jacobs, E., Foresta, F.D., Thiriart, C., Francotte, M., Thines, D., Wilde, M.D., 1989. Assembly and release of HIV-1 precursor $Pr55^{gag}$ virus-like particles from recombinant Baculovirus-infected cells. Cell 59, 103–112.

Goffinet, C., Allespach, I., Homann, S., Tervo, H.-M., Habermann, A., Rupp, D., Oberbremer, L., Kern, C., Tibroni, N., Welsch, S., Krijnse-Locker, J., Banting, G., Kräusslich, H.-G., Keppler, O.T., 2009. HIV-1 antagonism of CD317 is species specific and involves Vpu-mediated proteasomal degradation of the restriction factor. Cell Host Microbe 5, 285–297.

Göttlinger, H.G., Dorfman, T., Cohen, E.A., Haseltine, W.A., 1993. Vpu protein of human immunodeficiency virus type 1 enhances the release of capsids produced by gag gene constructs of widely divergent retroviruses. Proc. Natl. Acad. Sci. U. S. A. 90, 7381–7385.

Gupta, R.K., Hué, S., Schaller, T., Verschoor, E., Pillay, D., Towers, G.J., 2009. Mutation of a single residue renders human tetherin resistant to HIV-1 Vpu-mediated depletion. PLoS Pathog. 5 (5), e1000443.

Habermann, A., Krijnse-Locker, J., Oberwinkler, H., Eckhardt, M., Homann, S., Andrew, A., Strebel, K., Kräusslich, H.-G., 2010. CD317/tetherin is enriched in the HIV-1 envelope and downregulated from the plasma membrane upon virus infection. J. Virol. 84 (9), 4646–4658.

Hammonds, J., Wang, J.-J., Yi, H., Spearman, P., 2010. Immunoelectron microscopic evidence for tetherin/BST2 as the physical bridge between HIV-1 virions and the plasma membrane. PLoS Pathog. 6 (2), e1000749.

Harila, K., Prior, I., Sjöberg, E.M., Salminen, A., Hinkula, J., Suomalainen, M., 2006. Vpu and Tsg101 regulate intracellular targeting of human immunodeficiency virus type 1 core protein precursor $Pr55^{Gag}$. J. Virol. 80, 3765–3772.

Harila, K., Salminen, A., Prior, I., Hinkula, J., Suomalainen, M., 2007. The Vpu-regulated endocytosis of HIV-1 Gag is clathrin-independent. Virology 369, 299–308.

Hinz, A., Miguet, N., Natrajan, G., Usami, Y., Yamanaka, H., Renesto, P., Hartlieb, B., McCarthy, A.A., Simorre, J.-P., Göttlinger, H., Weissenhorn, W., 2010. Structural basis of HIV-1 tethering to membranes by the BST-2/tetherin ectodomain. Cell Host Microbe 7, 314–323.

Holm, K., Weclewicz, K., Hewson, R., Suomalainen, M., 2003. HIV-1 assembly and lipid rafts: $Pr55^{Gag}$ complexes associate with membrane-domains that are largely resistant to Brij 98, but sensitive to Triton X-100. J. Virol. 77, 4805–4817.

Hübner, W., McNerney, G.P., Chen, P., Dale, B.M., Gordon, R.E., Chuang, F.Y.S., Li, X.-D., Asmuth, D.M., Huser, T., Chen, B.K., 2009. Quantitative 3D video microscopy of HIV transfer across T cell virological synapses. Science 323, 1743–1747.

Ivanchenko, S., Godinez, W.J., Lampe, M., Kräusslich, H.-G., Eils, R., Rohr, K., Bräuchle, C., Müller, B., Lamb, D.C., 2009. Dynamics of HIV-1 assembly and release. PLoS Pathog. 5 (11), e1000652.

Iwabu, Y., Fujita, H., Kinomoto, M., Kaneko, K., Ishizaka, Y., Tanaka, Y., Sata, T., Tokunaga, K., 2009. HIV-1 accessory protein Vpu internalizes cell-surface BST-2/tetherin through transmembrane interactions leading to lysosomes. J. Biol. Chem. 284, 35060–35072.

Jouvenet, N., Neil, S.J.D., Bess, C., Johnson, M.C., Virgen, C.A., Simon, S.M., Bieniasz, P.D., 2006. Plasma membrane is the site of productive HIV-1 particle assembly. PLoS Biol. 4 (12), e435.

Jouvenet, N., Bieniasz, P.D., Simon, S.M., 2008. Imaging the biogenesis of individual HIV-1 virions in live cells. Nature 454, 236–240.

Jouvenet, N., Neil, S.J.D., Bess, C., Johnson, M.C., Virgen, C.A., Simon, S.M., Bieniasz, P.D., 2009. Broad-spectrum inhibition of retroviral and filoviral particle release by tetherin. J. Virol. 83, 1837–1844.

Kaletsky, R.L., Francica, J.R., Agrawal-Gamse, C., Bates, P., 2009. Tetherin-mediated restriction of filovirus budding is antagonized by the Ebola glycoprotein. Proc. Natl. Acad. Sci. U. S. A. 106, 2886–2891.

Kaplan, A.H., Manchester, M., Swanstrom, R., 1994. The activity of the protease of human immunodeficiency virus type 1 is initiated at the membrane of infected cells before the release of viral proteins and is required for release to occur with maximum efficiency. J. Virol. 68, 6782–6786.

Klimkait, T., Strebel, K., Hoggan, M.D., Martin, M.A., Orenstein, J.M., 1990. The human immunodeficiency virus type 1-specific protein vpu is required for efficient virus maturation and release. J. Virol. 64, 621–629.

Mangeat, B., Gers-Huber, G., Lehmann, M., Zufferey, M., Luban, J., Piguet, V., 2009. HIV-1 Vpu neutralizes the antiviral factor tetherin/BST-2 by binding it and directing its beta-TrCP2-dependent degradation. PLoS Pathog. 5 (9), e1000574.

Mansouri, M., Viswanathan, K., Douglas, J.L., Hines, J., Gustin, J., Moses, A.V., Früh, K., 2009. Molecular mechanism of BST2/tetherin downregulation by K5/MIR2 of Kaposi's sarcoma-associated herpesvirus. J. Virol. 83, 9672–9681.

McNatt, M.W., Zang, T., Hatziioannou, T., Bartlett, M., Fofana, I.B., Johnson, W.E., Neil, S.J.D., Bieniasz, P.D., 2009. Species-specific activity of HIV-1 Vpu and positive selection of tetherin transmembrane domain variants. PLoS Pathog. 5 (2), e1000300.

Mitchell, R.S., Katsura, C., Skasko, M.A., Fitzpatrick, K., Lau, D., Ruiz, A., Stephens, E.B., Margottin-Goguet, F., Benarous, R., Guatelli, J.C., 2009. Vpu antagonizes BST-2-mediated restriction of HIV-1 release via beta-TrCP and endo-lysosomal trafficking. PLoS Pathog. 5 (5), e1000450.

Miyagi, E., Andrew, A.J., Kao, S., Strebel, K., 2009. Vpu enhances HIV-1 virus release in the absence of BST-2 cell surface down-modulation and intracellular depletion. Proc. Natl. Acad. Sci. U. S. A. 106, 2869–2873.

Neil, S.J.D., Sandrin, V., Sundquist, W.I., Bieniasz, P.D., 2007. An interferon-alpha-induced tethering mechanism inhibits HIV-1 and Ebola virus particle release but is counteracted by the HIV-1 Vpu protein. Cell Host Microbe 2, 193–203.

Neil, S.J., Zang, T., Bieniasz, P.D., 2008. Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu. Nature 451, 424–430.

Ono, A., Freed, E.O., 1999. Binding of human immunodeficiency virus type 1 Gag to membrane: role of the matrix amino terminus. J. Virol. 73, 4136–4144.

Pais-Correia, A.-M., Sachse, M., Guadagnini, S., Robbiati, V., Lasserre, R., Gessain, A., Gout, O., Alcover, A., Thoulouze, M.-I., 2010. Biofilm-like extracellular viral assemblies mediate HTLV-1 cell-to-cell transmission at virological synapses. Nat. Med. 16, 83–90.

Perez-Caballero, D., Zang, T., Ebrahimi, A., McNatt, M.W., Gregory, D.A., Johnson, M.C., Bieniasz, P.D., 2009. Tetherin inhibits HIV-1 release by directly tethering virions to cells. Cell 139, 499–511.

Rong, L., Zhang, J., Lu, J., Pan, Q., Lorgeoux, R.-P., Aloysius, C., Guo, F., Liu, S.-L., Wainberg, M., Liang, C., 2009. The transmembrane domain of BST-2 determines its sensitivity to down-modulation by human immunodeficiency virus type 1 Vpu. J. Virol. 83, 7536–7546.

Sakuma, T., Noda, T., Urata, S., Kawaoka, Y., Yasuda, J., 2009. Inhibition of Lassa and Marburg virus production by tetherin. J. Virol. 83, 2382–2385.

Sato, K., Yamamoto, S.P., Misawa, N., Yoshida, T., Miyazawa, T., Koyanagi, Y., 2009. Comparative study on the effect of human BST-2/tetherin on HIV-1 release in cells of various species. Retrovirology 6, 53.

Schindler, M., Rajan, D., Banning, C., Wimmer, P., Koppensteiner, H., Iwanski, A., Specht, A., Sauter, D., Dobner, T., Kirchhoff, F., 2010. Vpu serine 52 dependent counteraction of tetherin is required for HIV-1 replication in macrophages, but not in ex vivo human lymphoid tissue. Retrovirology 7, 1.

van Damme, N., Goff, C., Katsura, C., Jorgensen, R.L., Mitchell, R., Johnson, M.C., Stephens, E.B., Guatelli, J., 2008. The interferon-induced protein BST-2 restricts HIV-1 release and is downregulated from the cell surface by the viral Vpu protein. Cell Host Microbe 3, 245–252.

Welsch, S., Keppler, O.T., Habermann, A., Allespach, I., Krijnse-Locker, J., Kräusslich, H.-G., 2007. HIV-1 buds predominantly at the plasma membrane of primary human macrophages. PLoS Pathog. 3 (3), e36.

Zhou, W., Parent, L.J., Wills, J.W., Resh, M., 1994. Identification of a membrane-binding domain within the amino-terminal region of human immunodeficiency virus type 1 Gag protein which interacts with acidic phospholipids. J. Virol. 68, 2556–2569.